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Cytoskeleton Dynamics in Drug-treated Platelets

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Abstract

Platelet activation is a key process in blood clot formation. During activation, platelets go through both chemical and physical changes, including secretion of chemical messengers and cellular shape change. Platelet shape change is mediated by the two major cytoskeletal elements in platelets, the actin matrix and microtubule ring. Most studies to date have evaluated these structures qualitatively, whereas this paper aims to provide a quantitative method of examining changes in these structures by fluorescently labeling the element of interest and performing single cell image analysis. The method described herein tracks the diameter of the microtubule ring and the circumference of the actin matrix as they change over time. Platelets were incubated with a series of drugs that interact with tubulin or actin, and the platelets were observed for variation in shape change dynamics throughout the activation process. Differences in shape change mechanics due to drug incubation were observable in each case.

Keywords

Platelet; activation; fluorescence imaging; cytoskeleton

Introduction

Platelets play a diverse set of roles in the body. The most well-characterized aspects of platelets are the roles they play in hemostasis and thrombosis, but they have also been implicated in processes such as inflammation and the migration of cancer cells [1-3].

In vessel injury, endothelial cells expose a variety of adhesion molecules and secrete small molecules. When platelets encounter the site of injury, the exposed adhesion molecules bind to the platelets and impede their travel. The binding of the adhesion molecules and the small molecules secreted by the endothelial cells initiate an activation cascade in the platelets that then leads to clot formation. Two main processes characterize platelet activation: (1) the secretion of small molecules and proteins and (2) a major cytoskeleton-mediated shape change [4,5]. Small molecule secretion functions to propagate the activation signal and initiate the wound healing process by other cells, and there has recently been significant progress in characterizing this secretion process [6-11]. Preliminary work has studied the accompanying shape change, wherein platelets undergo a major cytoskeletal rearrangement

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where the cell body swells up and then flattens out to form extensions called lamellipodia and filopodia, but the timeline of shape change has not been quantitatively evaluated [12]. The two main components that make up the platelet cytoskeleton are the microtubule ring and the actin matrix. Dissimilar to most cells, the microtubules in platelets form a circumferential loop at the outer edge of the platelet while the actin matrix is spread throughout the platelet [13, 14]. Our goal herein is to develop a way to make a direct connection between platelet secretion and morphological change, allowing both fundamental insight into platelet biology and critical studies about drug or disease effects on blood platelets. With the methods developed, this work demonstrates that as platelets in suspension undergo activation, both the actin matrix and microtubule ring decrease in size. As activation progresses, the actin matrix reaches a stable size whereas the microtubule ring shrinks to a certain extent and then breaks up into small microtubule fragments. It is possible to then compare the results from the imaging data with dynamic secretion measurements and obtain information by correlating the two. The results presented herein, when compared with data previously obtained, verify that the actin matrix acts as a barrier to dense-body granule secretion and that the microtubule ring is not involved in dense-body granule secretion [7].

Materials and Methods

Platelet Isolation

To isolate platelets, approximately 10-15 mL of rabbit blood was drawn from the midear artery of a rabbit after sedation according to IACUC protocol # 1311-31082A. The blood was centrifuged at 500 rcf with a brake speed of 0 for 15 minutes, at which point the supernatant, platelet rich plasma (PRP), was transferred to a clean centrifuge tube. The PRP was mixed with an equal volume of acid citrate dextrose solution (ACD; 85 mM trisodium citrate dihydrate, 66.6 mM citric acid monohydrate, 111 mM D-glucose) to prevent clotting during the platelet isolation. The PRP was then centrifuged at 750 rcf for 9 minutes to pellet the platelets, and the supernatant was removed. Next, the platelet pellet was resuspended in Tyrodes buffer (137 mM NaCl, 2.6 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 5.55 mM D-glucose, 5 mM HEPES, 12.1 mM $NaHCO_3$) and PGI_2 (0.5 μM). To ensure that the platelets had time for recovery, they were not used until 1 hour after isolation. Visual inspection of the platelets upon resuspension was performed to detect any morphological changes in the platelets, indicating activation. The platelet cell count was determined using a hemocytometer, with a typical isolated platelet concentration between $1\text{-}2 \times 10^8$ platelets/ml. The actin and microtubule experiments were performed on different days, and platelet preparations resulted in a lower concentration of platelets for the microtubule conditions.

Immunofluorescence Imaging

Immmunofluorescence imaging was performed on fixed platelets. Initially, platelets, at a concentration of 1×10^7 platelets/mL, were activated using human thrombin (5 units/mL, Sigma-Aldrich) at room temperature. Aliquots from the activated PRP were removed at 50 s intervals and activation was quenched by addition of the PRP to 8% formaldehyde in Tyrodes buffer [15]. Fixation was allowed to proceed for 20 minutes in the same 8% formaldehyde solution, after which the platelets were pelleted by centrifuge for 5 minutes at

2500 rcf. The fixative solution was removed, and the platelets were resuspended in 0.1% Triton X-100 in Tyrodes buffer (Sigma Aldrich) containing 0.1 mM EGTA (Sigma Aldrich). After 10 minutes of permeabilization, the platelets were again washed via pelleting, supernatant removal, and resuspension. Next, the platelets were incubated in a 1% BSA solution in Tyrodes buffer for 30 minutes to block nonspecific antibody binding. After another wash step, the platelets were incubated with either a Cy3-conjugated anti-β-tubulin-antibody (Abcam, ab11309) to label the microtubule ring, with the antibody diluted 1:100 in 1% BSA in Tyrodes, or a FITC-conjugated anti-β-actin-antibody (Abcam ab11005) to label the actin matrix, with the antibody diluted 1:250 in 1% BSA in Tyrodes buffer. The antibody incubation was performed overnight at 4°C. Finally, the platelets were washed again and allowed to settle onto poly-L-lysine-coated coverslips (1 μg/mL, 0.1% w/v poly-L-lysine in H_2O, Sigma Aldrich) for imaging.

The fixed and labeled platelets were imaged using a Nikon Eclipse TE2000-U microscope and a Photometrics QuantEM:512SC camera. A 100x 1.40 NA oil immersion objective was used to obtain sufficient magnification of the platelets to enable clear visualization of the microtubule ring.

Drug Treatment

Drug-treated platelets were incubated with cytochalasin D, latrunculin A, vincristine, or paclitaxel (Sigma Aldrich) at a concentration of 10 μM for 45 min at room temperature prior to activation. The concentration used was chosen based on previous work examining the platelet cytoskeleton [7, 16]. As cytochalasin D, latrunculin A, and paclitaxel are insoluble in water, they were first dissolved in DMSO. As a control, platelets were also incubated with an equivalent DMSO concentration prior to activation to account for any effects DMSO itself might have on platelets [17].

Image Analysis

For each condition, approximately 20 82 μm-by-82 μm fluorescence images were recorded, with several platelets visible in each image. Sample shape change progressions are shown in Figures 1 and 3 for the microtubule ring and actin matrix, respectively (For drug treatment progressions see Electronic Supplemental Material Fig. S1 –S6). For the microtubule ring-stained platelets, the diameter of each microtubule ring was measured at three locations to account for any non-circular character of the microtubule ring (Figure 2A). For the actin-treated platelets, the circumference of the actin matrix was measured. An ellipse was drawn inside the fluorescently labeled actin matrix, touching but not exceeding the edges (Figure 4A). For each condition, 25 platelets were measured. By determining the average measured value for each time point, a plot was created showing the change in diameter or circumference over time. Each plot was fit using a one-phase decay curve. Statistical analysis was performed using Graphpad Prism. One-way ANOVA was used to compare the shape change as a function of time. A comparison of fits was also used to determine significance between the various treatments. Any $p < 0.05$ was considered significant.

Results and Discussion

There are several pharmacological agents with modes of action based on cytoskeletal elements; these drugs present a perfect platform to prove the utility of these platelet imaging analyses. To disrupt microtubule dynamics, platelets were incubated with either vincristine or paclitaxel. At the concentrations used, paclitaxel is known to stabilize microtubules, and vincristine is known to cause destabilization of microtubules [18]. The same procedure was applied to examine the role of the actin matrix in platelet secretion wherein platelets were incubated with either cytochalasin D or latrunculin A, both of which are known to inhibit polymerization of actin filaments. The modes of inhibition vary between cytochalasin D and latrunculin A, where the former binds to the filament and prevents addition of monomers while the latter binds to the monomer to prevent addition to the filament [19, 20].

Microtubule results

Image Processing

The labeled microtubules exhibited well-defined fluorescence, clearly showing that the labeled structures formed a ring. The rings were not completely circular, many being ellipsoidal in shape. To get an accurate measurement of the size change, the diameter of the microtubule ring was measured three times, where the longest, shortest, and an intermediate diameter were chosen (Figure 2A). In addition, platelets that appeared to have settled on the coverslip at an angle were not measured, as their dimensions were skewed, appearing thin and long.

No Treatment

Untreated (control) platelets showed a change in microtubule ring diameter from 3.3 ± 0.1 μm to 1.7 ± 0.1 μm during the 240 s time course following activation with thrombin. The primary diameter change occurred over the first 40 s of activation ($p < 0.0001$); while the diameter appears to decrease over the remaining 150 s, the changes are not statistically significant (Figure 2B).

DMSO

A control was also performed using DMSO, a necessary reagent to dissolve the paclitaxel. The diameter of the DMSO-treated platelets started and ended at 3.4 ± 0.1 μm and 1.7 ± 0.1 μm, respectively (Figure 2B). These diameters were not statistically different from those measured from the non-treated platelets ($p > 0.05$). However, during activation, the DMSO-treated platelets exhibited a more start-stop approach to microtubule shrinkage, where the diameters had statistically significant decreases between 0 and 40 s ($p < 0.0001$) and 90 and 140 s ($p < 0.05$). This was further exemplified by the fact that one phase exponential fit tested via ANOVA did not fit both data sets ($p < 0.05$).

Paclitaxel

The paclitaxel-treated platelets were statistically compared to the DMSO-treated platelets as the paclitaxel solutions were made up in DMSO. The paclitaxel-treated platelets started off with a smaller ring diameter, 3.1 ± 0.1 μm, than the DMSO-treated platelets. After

activation, the paclitaxel-treated platelets initially exhibited a slower decrease in ring diameter compared to the DMSO-treated platelets ($p < 0.001$). Around 140 s however, the shrinkage dynamics of the microtubule ring in the paclitaxel-treated platelets became greater in magnitude than those of the DMSO-treated platelets, resulting in a final diameter of 1.5 ± 0.0 μm (Figure 2C). Similar to the DMSO-treated platelets, the primary shape change occurred between 0 and 40 s ($p < 0.0001$) and 90 and 140 s ($p < 0.05$) of activation.

Vincristine

Treating platelets with vincristine resulted in the destruction of the microtubule ring dynamics. These platelets had microtubule rings that started off with an average diameter of 2.3 ± 0.1 μm and ended at a diameter of 2.5 ± 0.1 μm. The diameter fluctuated throughout the 240 s with a low of 2.2 ± 0.0 μm at 90 s and a high of 2.5 ± 0.1 μm at 240 s.

Actin results

Image Processing

Labeling of the actin matrix is not as straightforward as labeling the microtubule ring because the cytoskeletal element is not as well defined. The actin-based fluorescence images showed diffuse fluorescence that appeared throughout the platelet. There were some structures visible in the form of dark spots within the fluorescent area, possibly resulting from granules, but they were uncommon and thus left uncharacterized (Figure 5). Like the microtubule rings, the fluorescent structure was not uniformly circular in shape and so it was necessary to determine the best measurement to characterize the structure. The methods tried include: fitting a circle or ellipse to the exterior or interior of the actin matrix or tracing the edges of each platelet. While tracing the edges initially appeared to be the best method, it became apparent that the time required to accurately trace the edge of each actin matrix was substantial. In addition, the edges were not always clear in the images, making this method somewhat subjective. More of the images exhibited platelets with an elliptical shape than a round shape, and so using the ellipse to approximate the platelet size was found to be most efficient and effective. When comparing measurements made on the exterior or interior of the fluorescent area, the interior measurements visually resulted in a closer fit to the true circumference than the exterior measurements. Thus a best-fit ellipse was used to approximate the actin matrix circumference, though this may result in a slight underestimation of total actin coverage.

No Treatment

The platelets that were not subjected to drug treatment showed a change in actin matrix circumference from 10.9 ± 0.3 μm to 9.1 ± 0.2 μm over the 240 s imaged (Figure 4B). The key characteristic found for actin matrix shape change was that the majority of the shrinkage occurs within the first 40 s after platelet activation. While the size did not appear to be completely static during the later time points, the differences in the circumference were not statistically significant ($p > 0.05$).

DMSO

Similar to the microtubule disruption experiments, a control set of platelets were treated with DMSO as both latrunculin A and cytochalasin D are insoluble in water. The DMSO-treated platelets exhibited a more gradual decrease in circumference compared to the untreated platelets, going from 9.5 ± 0.4 μm to 8.2 ± 0.2 μm during the time monitored (Figure 4B). The decrease in size occurred over the first 90 s rather than the first 40 s in the untreated platelets ($p \leq 0.05$). Similarly however to the untreated platelets, later changes were not statistically significant ($p > 0.05$). It is also important to note that, in addition to the change in time course, the DMSO-treated platelets started and ended up with a smaller circumference than the untreated platelets, despite the low DMSO concentrations used, indicating that the DMSO does influence the normal actin dynamics.

Latrunculin A

The latrunculin A-treated platelets started off the same size as the DMSO-treated platelets but did not change size in a statistically significant manner at any point during activation ($p > 0.05$). The circumference varied slightly, within a range in 0.5 μm where they were at their smallest at the 0 s time point and their largest during the 90 s time point, going from 9.6 ± 0.2 μm to 9.9 ± 0.2 μm (Figure 4C).

Cytochalasin D

The decrease in size of the cytochalasin D-treated platelets occurred faster than that of the DMSO-treated platelets, where they reached a stable size within the first 40 s of activation. In addition, the cytochalasin D-treated platelet circumferences started off 1.5 μm larger than the DMSO-treated platelets and ended 0.5 μm larger, going from 11.0 ± 0.3 μm to 9.1 ± 0.2 μm, despite the DMSO-treated platelets being larger at the 40 s time point (Figure 4D).

Based on the fact that there are some significant changes in platelet cytoskeletal elements upon drug treatment, we considered correlation between these changes and platelet secretion of chemical messenger species. To this end, the microtubule ring and actin matrix dynamics were compared to previously published data showing the release of serotonin from platelets incubated with the same drugs at the same doses and incubation times. Previous results showed that when the platelets were incubated with the microtubule destabilizing drugs, the serotonin secretion from dense-body granules was not affected. However, when the actin matrix was disrupted, serotonin secretion was affected. With this previously published information in concert with the two data sets presented herein, we can see that the microtubule ring is not involved in the dense body granule release process despite the changes that were observed during activation [7]. Perhaps the microtubule ring behavior is more closely associated with alpha granule release (not assessed here) or shrinks to the inside of the platelet to minimize interactions with the granules during activation [21]. Future work will explore these hypotheses by performing simultaneous imaging of the alpha granules and the cytoskeletal elements.

Conclusions

Here, the dynamic changes of the cytoskeleton have been quantitatively tracked. Changes in the microtubule ring were tracked during the activation process by measuring the diameter of the ring at various time points. The images show that during activation, the microtubule ring first exhibits a decrease in diameter with retention of its circular or elliptical shape. However as activation proceeds, the microtubule ring begins to break apart into distinct pieces. The dynamics of the actin matrix are more difficult to quantify due to the more diffuse and abstract feature shape; however, changes in the actin matrix were effectively tracked by placing a best fit ellipse into the fluorescent area representing the labeled actin to measure the circumference. Like the microtubule ring, the actin matrix first decreases in size. After the initial decrease in circumference, at about 40 s post-activation the actin matrix reaches a steady circumference that remains throughout the rest of the activation process.

Future studies will involve tracking the cytoskeleton dynamics of live rather than fixed cells and a further exploration of the dynamics within the first 40 s of activation, where it became apparent through this work that the majority of cytoskeletal changes are occurring. Also, various microscopy techniques will be applied in an effort to increase the spatial resolution so that features within the actin matrix are more visible. Through live cell imaging techniques with increased resolution it will be possible to further elucidate the intertwining roles of the platelet cytoskeleton and granule release.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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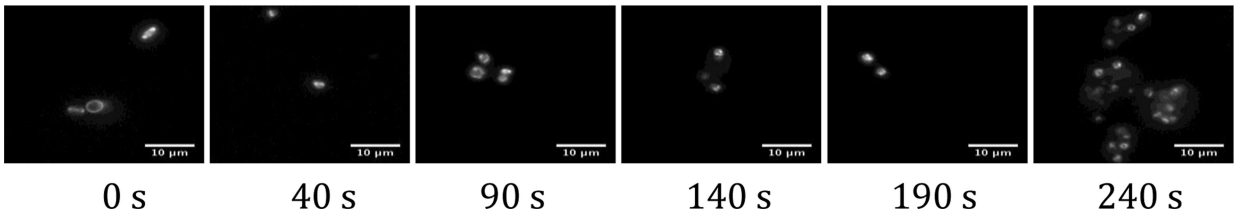

Figure 1.

Microtubule ring – No treatment. Timeline of microtubule ring shrinkage dynamics over the course of 240 s following activation. The ring structure of the microtubules can be seen initially but becomes less well-defined as activation progresses. Scale bar 10 μm.

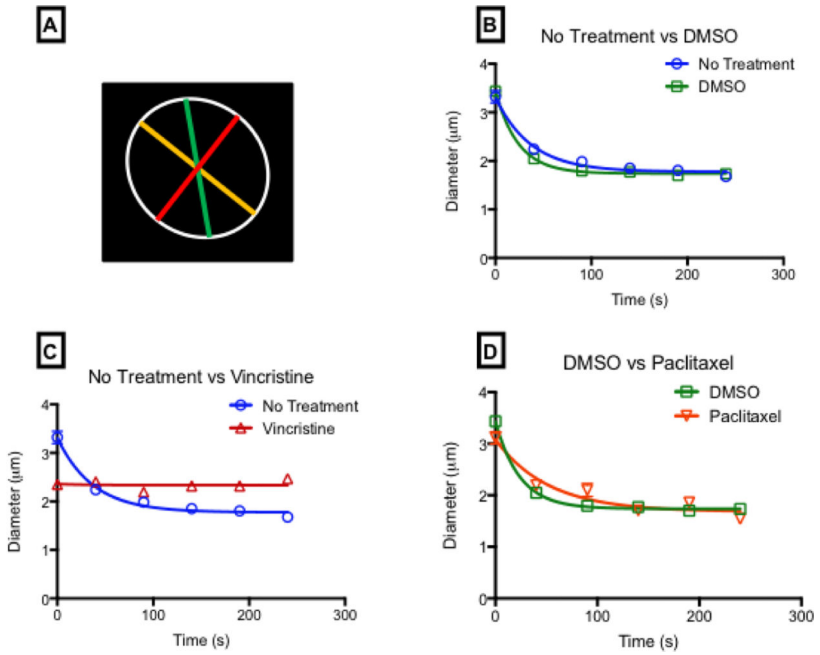

Figure 2.

A) The microtubule ring of each platelet was measured by taking the diameter of the ring at the smallest, largest, and intermediate lengths. B-D) Comparison of the microtubule ring shrinkage curves for non-treated and DMSO-treated platelets, DMSO-and paclitaxel-, and DMSO- and vincristine-treated platelets, respectively. Error bars indicate standard error of the mean (SEM).

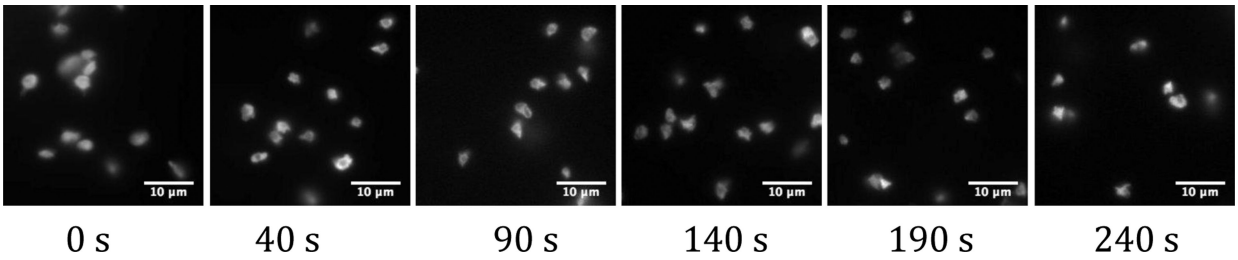

Figure 3.

Actin matrix – No treatment. Timeline of actin matrix shrinkage dynamics over the course of 240 s following activation. Scale bar 10 µm.

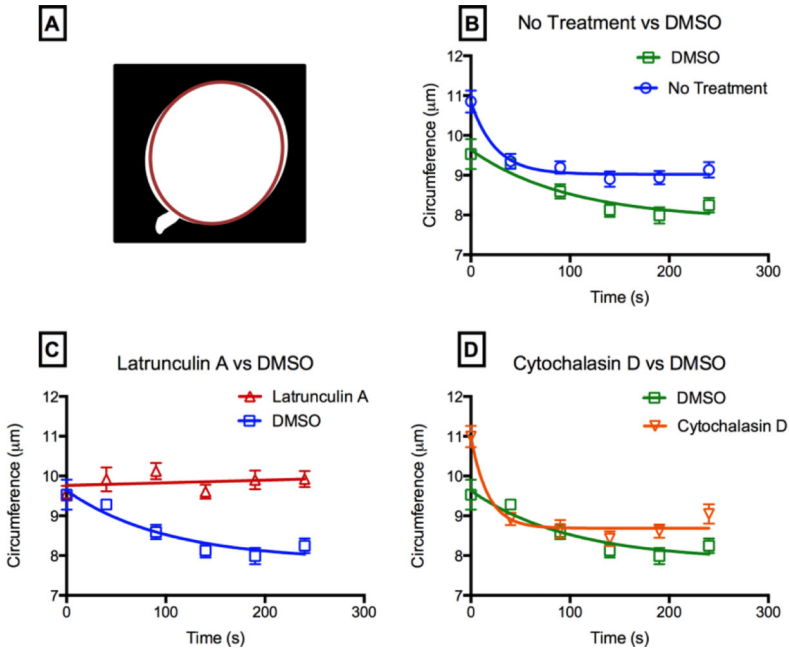

Figure 4.

A) The actin matrix of each platelet was measured by using the best fit ellipse to measure the circumference. B-D) Comparison of the actin matrix shrinkage curves for non-treated and DMSO-treated platelets, DMSO- and latrunculin A-, and DMSO- and cytochalasin D-treated platelets, respectively. Error bars indicate SEM.

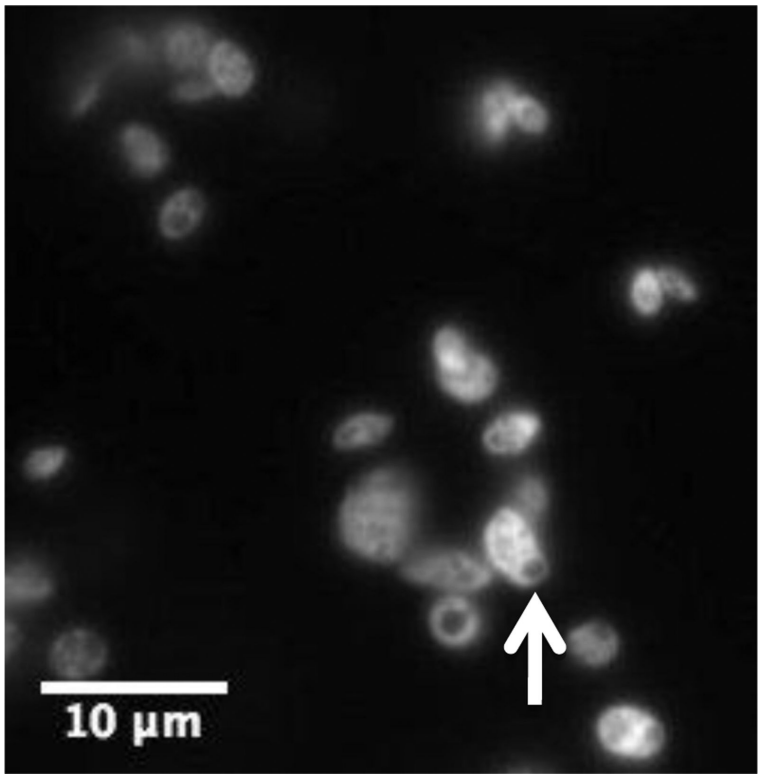

Figure 5.
Dark spots seen throughout the platelet during actin matrix labeling may be due to granules within the platelet. Platelets with these features exhibited one or more spots.